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Induction of Mutations in *Drosophila melanogaster gypsy* Retroelements by Modulation of Intracellular Deoxynucleoside Triphosphate Pools In Vivo▽

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The retroviral mutation rate is susceptible to a number of variables, including the balance between intracellular deoxynucleoside triphosphate (dNTP) pools. While this follows from tissue culture studies, the issue has never been addressed directly in vivo. To explore this question in a tractable experimental system, we analyzed the impact of thymidine treatment on the synthesis of *gypsy* retroelement cDNA from *Drosophila melanogaster* during development through to hatching. The mutation frequency was enhanced approximately 16-fold over the levels seen in the experimental background. Due to the lack of proofreading, these *gypsy* elements represent hypervariable loci within the *Drosophila* genome, suggesting that dNTP pool imbalances in vivo are mutagenic.

Intracellular deoxynucleoside triphosphate (dNTP) pool imbalances during replication are generally mutagenic in vitro (8, 9, 16, 17, 18, 29). The allosteric enzyme ribonucleotide reductase ensures a pool of deoxynucleoside diphosphates that are further phosphorylated to yield dNTPs, dUTP being subsequently degraded by dUTPase (21, 25). Although the synthesis of dTTP is independent of the presence of ribonucleotide reductase, it has a negative effect on CDP reduction by this enzyme and results in a detectably lower intracellular concentration of dCTP (2, 21, 25). Thymidine (Thd) is therefore efficient at influencing dNTP pools, as it not only increases the dTTP concentration but also decreases the concentration of dCTP. Furthermore, the enhanced imbalance in the [dTTP]/[dCTP] ratio is conducive to the formation of $G_{template}$:T pairs, the most stable of all mismatches (5, 20).

The replication of some viruses is susceptible to modulation by thymidine and hydroxyurea, both of which impinge on dNTP synthesis (19, 28). In the case of retroviruses, treatment by thymidine would result in rG:dT mismatches that go uncorrected because, like that of all RNA viruses, cDNA synthesis occurs in the absence of proofreading (3, 13, 14, 19, 31). Hence, retroelements represent hypersensitive loci.

The *Drosophila melanogaster gypsy* endogenous retrovirus has three large open reading frames (ORFs) bounded by two long terminal repeats (12, 15, 26). Despite being an endogenous element, *gypsy* is infectious (6, 30). The *flamenco* (*flam*) locus, located on the X chromosome, is a major regulator of *gypsy* (24). In restrictive strains, functional *flam* alleles maintain *gypsy* proviruses in a repressed state. By contrast, in permissive strains, e.g., the MG strain, proviral amplification results from infection of the female germ line and subsequent insertions into the chromosomes of the progeny (23).

We found that the addition of 1 mM thymidine to standard *Drosophila* medium inhibited egg laying. To overcome this problem, between 300 and 500 *D. melanogaster* MG permissive strain eggs were placed on standard medium (4) and allowed to hatch and grow to adults (Fig. 1A). The medium was supplemented with 1 mM Thd, 1 mM Thd–1 mM deoxycytidine (dCyd)–200 μM tetrahydrouridine (THU), or 1 mM dCyd–200 μM THU. dCyd was added to counter the depletion of dCTP resulting from Thd treatment. THU was used to protect against spontaneous deamination of dCyd by deoxycytidine deaminases.

In the presence of 1 mM Thd, 75% of the eggs did not hatch (Fig. 1B). The remaining 25% became young flies, albeit requiring more development time than the nontreated eggs (data not shown). The viability of eggs treated with 1 mM dCyd–200 μM THU was significantly higher than that of eggs placed on medium supplemented with 1 mM Thd. Eggs placed on medium supplemented with 1 mM Thd–1 mM dCyd–200 μM THU were slightly less viable than the eggs in the control group (Fig. 1B). These data show that intracellular dCTP depletion following Thd addition can be substantially abrogated by the addition of dCyd.

It was previously shown that mobilization of gypsy in the progeny of MG females correlates with higher levels of full-length gypsy RNA in the ovaries (23). It is at this stage that reverse transcription and dNTP incorporation occur. As *gypsy* cDNA synthesis and transposition occurs in MG female germ cells, genomic DNA extraction and PCR were done on F_1

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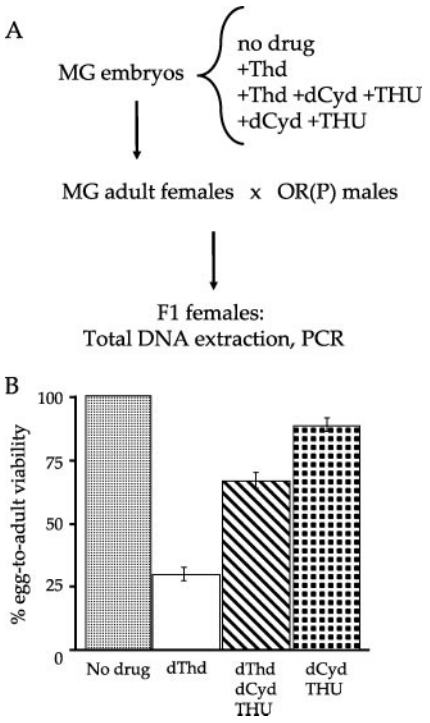

FIG. 1. Experimental procedures and modulation of *D. melanogaster* egg viability by modulation of DNA precursor metabolites. (A) MG embryos were raised on standard *Drosophila* medium with or without drugs. Transposed proviral *gypsy* copies were then analyzed in the progeny of MG females. Thd and 2′-dCyd were purchased from Sigma, and THU was purchased from Calbiochem. (B) Restricting *D. melanogaster* egg-to-adult viability by treatment with 1 mM Thd as well as reversing the effect by addition of 1 mM dCyd (plus 200 μM THU). Data are expressed as the percentage of young adult numbers divided by initial egg number relative to control (no drug) egg-to-adult viability. Bars represent 95% confidence intervals.

adults resulting from individual crosses between the MG females (treated and untreated) and OR(P) males, which are devoid of active *gypsy* proviruses (1). As a result, all F_1 progeny harbored MG-derived *gypsy* elements in all cells (Fig. 1A). In order to detect de novo-synthesized *gypsy* cDNA, a genetic screening was set up based on *Escherichia coli* β-galactosidase complementation (31). Given the thymidine treatment protocol, uncorrected rG:dT mismatches should show up as G→A transitions. A small locus bearing tryptophan codons was amplified from the *gypsy* ORF1 (Fig. 2A). As tryptophan is encoded by the single codon TGG, accumulation of G→A transitions yields one of three stop codons (TGA, TAG, or TAA). When cloning in frame into the β-galactosidase gene is performed, mutants are scored as white plaques and wild-type sequences as blue plaques.

Total DNA was extracted from two 3-day-old F_1 females per regimen. As many deleted *gypsy* elements are present in the genome of *D. melanogaster* (10), a first-round PCR of nearly full-length elements was performed using primers JF1 (5′CA ACCAACAATCTGAACCCACCAAT; positions 489 to 513, accession number M12927) and JF2 (5′TGGCTGCTTGCTT AGTGTTTTCCTT; positions 6691 to 6667). Nested PCR was performed with primers MC1 (5′GCGGAATTCTTACGCCT GTTGAGGCAA; positions 1644 to 1661) and MC2 (5′GCG AAGCTTACCTACCACTAATGCAA; positions 1687 to 1703). Standard PCR conditions and *Pfu* polymerase were used. The amplified products were cloned in frame using the *lacZ* α fragment of M13mp18 via EcoRI and HindIII (sites underlined). *E. coli* XL1-Blue was transformed and plated on 8% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plus IPTG (isopropyl-β-D-thiogalactopyranoside) indicator plates. DNAs from white plaques were purified and sequenced by a previously published method (31).

An average of 1,200 colonies were obtained from each DNA sample. For the progeny of untreated strain MG mothers (flies D1 and D2) as well as those from MG mothers treated using dCyd plus THU (flies D5 and D6), a single white plaque was found which reflected a single point mutation in a tryptophan codon (Fig. 2B). This meant that the mean background mutant and mutation frequencies were $\sim 3.5 \times 10^{-4}$ and $\sim 1.4 \times 10^{-5}$, respectively, which are not statistically different ($P = 0.55$; Fisher's exact test). By contrast, for both flies from MG mothers that were grown on the 1 mM Thd regimen (flies D3 and D4), a small number of white clones were identified (mean frequency, $\sim 6.5 \times 10^{-3}$). All but two clones harbored single G→A transitions, as would be expected if dTTP were incorporated opposite rG during minus-strand DNA synthesis. The mean point mutation frequency of 2.2×10^{-4} is approximately 16-fold greater than that seen with the untreated controls ($P = 0.0002$; Fisher's exact test). It is noteworthy that no mutants or mutations were detected for flies treated with the regimen of Thd plus dCyd plus THU (flies D7 and D8; Fig. 2B), indicating that dCyd was able to counter the mutagenic effects of Thd.

In reports going back more than 30 years it was found that the administration of thymidine to culture medium of *D. melanogaster* resulted in discernible traits, most notably cuts in the wing margins and wing-vein abnormalities (22). However, mutagenic effects at the nuclear level were not reported. It is probable that some mutations arising from dNTP pool imbalances must occur in chromosomal DNA and escape mismatch repair. Following Thd treatment, the *gypsy* mutation rate was increased 16-fold to $\sim 2.2 \times 10^{-4}$/base/cycle. It may be supposed that the treatment increased the germ line mutation rate. In keeping with this is the observation that a large fraction of eggs died on 1 mM thymidine-supplemented medium. Accordingly, it is anticipated that submillimolar concentrations of Thd will also prove to be mutagenic.

It is interesting to transpose these findings to the setting of the mammalian cell, for which there are far more data. A recurrent observation is the bias towards GC→AT transitions in comparisons of mutated cancer-associated genes or deleterious alleles to "wild-type" alleles and of pseudogenes to their cognate genes (7, 11). While a proportion of these results must be attributed to deamination of methylated deoxycytidine in the context of CpG, there remains an excess GC→AT bias even when CpG motifs are accounted for (27). The present work suggests that increases in the intracellular dTTP levels may be a viable mechanism in vivo underlying a fraction of GC→AT transitions. In conclusion, *D. melanogaster gypsy* elements represent a tractable model for investigation of the

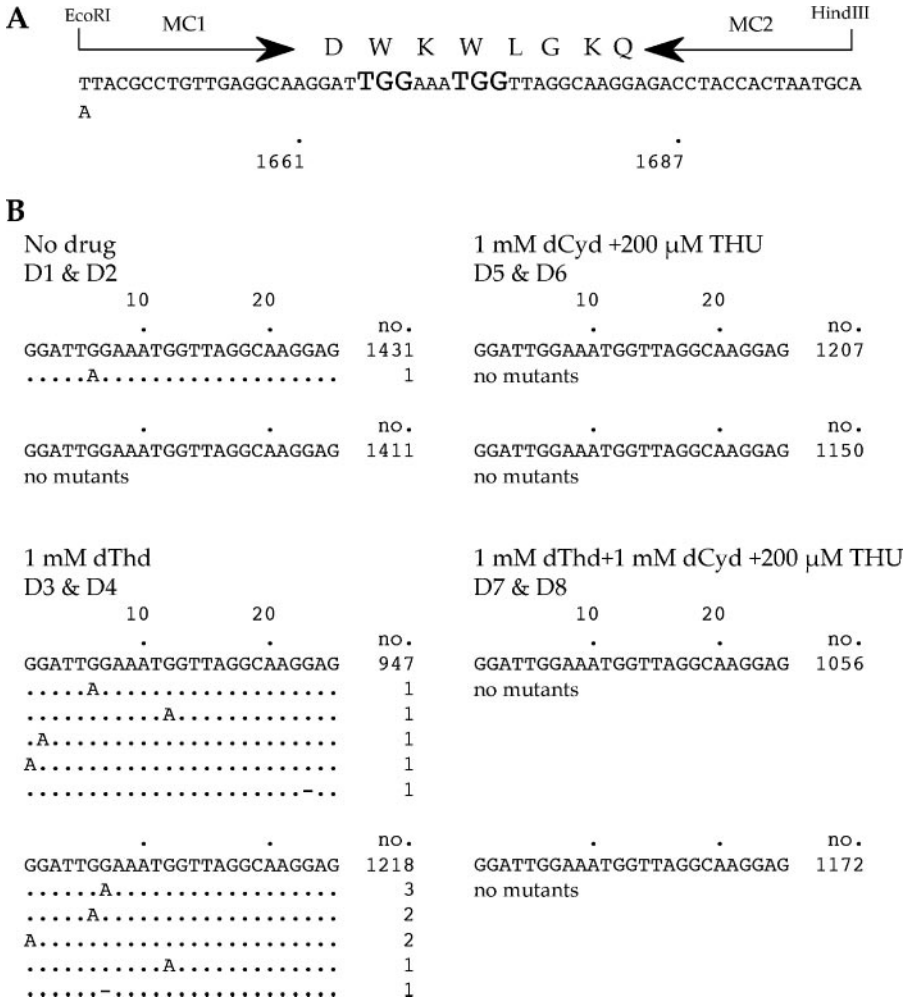

FIG. 2. A tryptophan codon trap for *gypsy* G→A transitions in vivo. A) Sequence of the 60 bp tryptophan trap in *gypsy* retroelement ORF1. The 25 bp insert between primers MC1 and MC2 encodes two tryptophan codons (larger type). B) Trap sequences corresponding to white M13mp18 plaques from total DNA from two mature flies for each treatment. Flies are numbered D1 through D8. Sequence differences are indicated; dots indicate identity, while dashes indicate deletions. Plaque frequencies are shown to the right of the sequences. Four clones representing two distinct sequences harbored G→A transitions outside of the two tryptophan codons. To be sure that they did not represent some sort of false-negative white clones, their insertions were reamplified using the MC1-MC2 primer pair, re-cloned, and resequenced. Both yielded a clear white phenotype, while sequencing confirmed the initial sequence (data not shown).

relationship between small molecules, dNTP pool imbalances, and mutation in vivo.

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